Florida Fish and Wildlife Conservation Commission
Fish and Wildlife Research Institute
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Microscopic Analysis of Microalgae – KPP Counting
(*Karenia brevis*, *Pseudo-nitzschia* spp., and *Pyrodiniuum bahamense*)

Revised: May 2020
NOTE: This version supersedes all previous versions

Materials & Equipment:

- 5 mL polystyrene disposable standard serological pipets graduated in 1/10 mL (Fisher cat# S68228C)
  NOTE: DO NOT REUSE PIPETS
- Pipet dispenser (Fisher cat# 13-681-102A or 13-681-102A)
- Lab-Tek™ 2-chambered Coverglass System (Fisher cat# 12-565-336), aka NUNCs
  NOTE: cat# 12-565-471 if the former is out of stock
- Lugols Stock Solution (prepared in-house at the EM lab; see specific procedure)
- Inverted microscope with objective/ocular combination to magnify 100-200x
- Versa-Clean (Fisher cat# 04-342)
- Foam-Tipped Swabs (Fisher cat# 14-960-3J)
- Counter or multi-unit counter for counts of more than one species
- Count data sheets to always include the following:
  HABW#, collection date, collection location, geographic coordinates, chamber number, volume settled, analysis date, analyst, genus and species, raw cell count numbers and calculated cells/liter concentrations.

Preserved vs. Live Phytoplankton Samples

Cells need to be preserved (not moving, not reproducing) to be examined (enumerated) by microscopy. Examination of phytoplankton samples that have been preserved at the time of sampling have two main advantages over enumeration of samples received live: they do not have to be counted immediately and they are a better representation of the community at the exact time of sampling. However, only live samples can be used for other procedures in the lab such as: toxin analysis, isolation into cultures, pigment and nutrient analysis, to mention a few. Because the community in a live sample is changing “as we speak”, their analysis takes priority over preserved samples. If the analysis of a live sample is not possible upon arrival (within hours), an aliquot of the sample must be preserved ASAP.

Counting preserved samples

There are several types of fixatives that can be used to preserve microalgae cells, each with their own advantages/disadvantages according to the objective of the study and the target organism. Lugol’s solution is recommended for most flagellates and diatoms and has the added advantage of not requiring its use under a fume hood, as some other common fixatives do (e.g., glutaraldehyde, formaldehyde).

1. Gently invert the sample at least 20 times. Remove the cap and immediately pipet 3 mL of sample from half-way within the bottle. Dispense the sample in one of the two chambers of the NUNC system, put the lid on, and

let the chamber sit in its own tray, in the dark (cover the tray with aluminum foil). Allow the sample to settle for at least 30 minutes before counting. If the sample is not counted right away, keep the chamber stored in the dark. If the settled sample is not examined within 48 hours, discard chamber and settle a fresh aliquot of material.
NOTE: use only one pipet per sample, that is, discard after use.
2. Routine cell counts are performed under ~ 200x final magnification. To analyze samples with cells in low concentrations, move along each horizontal transect, row by row, to cover the entire chamber.
3. To obtain the number of cells/Liter, when counting the 3 ml aliquot, divide the number of cells counted in the entire chamber by 3, then multiply by 1,000.
4. To analyze samples with higher cell concentrations, refer to the procedure described in Table 1. In such cases, a reduced portion of the chamber is scanned (horizontal transects or random fields of view) and the cell number found is extrapolated to the whole chamber counting area. The number (multiplication factor) used to make this extrapolation is particular to each microscope and will depend on the size of the area actually scanned (number of transects/fields). A table with multiplication factors is found next to each microscope. If visual inspection under lower magnification indicates cell distribution is not random, make sure multiple transects represent both the edge and the middle of the chamber (but do not visually choose the transect!). Once you calculate the total number of cells for the chamber, obtain the number of cells/Liter following step (3).

Counting live samples
1. Add a drop of Lugol's solution to the chamber well. Gently invert the sample at least 20 times. Remove the cap and immediately pipet 3 mL of sample from half-way within the bottle. Dispense the sample in one of the two chambers of the NUNC system, put the lid on, and let the chamber sit in its own tray, in the dark (cover the tray with aluminum foil). Allow the sample to settle for at least 30 minutes before counting. If the sample is not counted right away, keep the chamber stored in the dark. If the settled sample is not examined within 48 hours, discard chamber and settle a fresh aliquot of material.
2. Follow steps #2 through #4 from previous section.

NOTES:
(a) The counting unit is the cell, not a chain of cells; that is, every single, individual cell must be counted.
(b) While extrapolating numbers, keep track of ~3 decimal points; but record final cell/Liter with only significant digits. For example: 667; 7,600; 18,600; 1,857,000 cells/Liter
(c) Record data in permanent ink on count datasheets.
(d) Lugol's solution makes the surface of the cells sticky. They will stick to the sample bottles and to the NUNC chambers. To prevent sample cross contamination:
- Wash the sample bottles with a Versa-Clean solution between uses.
- Clean the chambers the day of use; if not possible, rinse with tap water and soak in diluted Versa-Clean solution.
- To clean NUNC chambers, use foam tipped swabs, rinse 3x with tap water and then 3x with distilled water; let then air dry facing down.

How many cells to count?

In order to obtain a statistically robust result for phytoplankton cell concentration, it is necessary to consider a certain number of counting units (cell, chain, colony, filament). The number of cells to count will depend on the precision desired, usually expressed as the 95% confidence limit as a proportion of the mean. The relationship between the confidence range and the number of cells counted is not linear. It is given by the following equation:

confidence range/limit (or ± error expressed in %) = (2 * 100) / √number cells counted

If we desire 20% precision in our results, it is necessary to count 100 cells (see table attached). That means that the estimated cell count may actually fall anywhere between 80 and 120 cells.
In practice, due to time constraints, we do the best we can to achieve a pre-established goal, so that we can standardize the error across samples analyzed. To standardize the best we can, follow the procedure in the table below. It is good practice to scan the chamber as you move to the starting point to assess overall distribution of cells. It saves time to make the decision of how to count that particular sample.

Table 1. How many cells to count?
In an effort to reach as close as possible to 100 cells/count, if you find:

# cells in 1st transect	**YOU SHOULD COUNT**
<10	whole chamber
10	whole chamber
20	5-7
30	4-5
40	3-4
50	2-3
60	2 (if 2nd transect is <54 or >66, count 3rd transect)
70	2 (if 2nd transect is <63 or >77, count 3rd transect)
80	2 (if 2nd transect is <72 or >88, count 3rd transect)
90	2 (if 2nd transect is <81 or >99, count 3rd transect)
100	2 (if 2nd transect is <90 or >110, count 3rd transect)
> 100	@20x, start with 3 fields of view; if number of cells vary more than ± 20%, keep adding fields of view up to 8-10 fields of view and discard lowest and highest counts
	@40x, same as above, with a minimum of 5 fields of view

Relationship between cell count and the confidence range at 95% significance level (Edler & Elbrächter, 2010)

confidence range/limit (or ± error expressed in %) = (2 * 100) / √number cells counted

number cells counted	confidence interval %	cels/L (NUNC, 3mL)	total count error	min	max
1	200	333	667	-	667
2	141	667	943	195	1,138
3	115	1,000	1,155	423	1,577
4	100	1,333	1,333	667	2,000
5	89	1,667	1,491	921	2,412
6	82	2,000	1,633	1,184	2,816
7	76	2,333	1,764	1,451	3,215
8	71	2,667	1,886	1,724	3,609
9	67	3,000	2,000	2,000	4,000
10	63	3,333	2,108	2,279	4,387
11	60	3,667	2,211	2,561	4,772
12	58	4,000	2,309	2,845	5,155
13	55	4,333	2,404	3,131	5,535
14	53	4,667	2,494	3,419	5,914
15	52	5,000	2,582	3,709	6,291
16	50	5,333	2,667	4,000	6,667
17	49	5,667	2,749	4,292	7,041
18	47	6,000	2,828	4,586	7,414
19	46	6,333	2,906	4,880	7,786
20	45	6,667	2,981	5,176	8,157
21	44	7,000	3,055	5,472	8,528
22	43	7,333	3,127	5,770	8,897
23	42	7,667	3,197	6,068	9,265
24	41	8,000	3,266	6,367	9,633
25	40	8,333	3,333	6,667	10,000
26	39	8,667	3,399	6,967	10,366
27	38	9,000	3,464	7,268	10,732
28	38	9,333	3,528	7,569	11,097
29	37	9,667	3,590	7,872	11,462
30	37	10,000	3,651	8,174	11,826
31	36	10,333	3,712	8,477	12,189
32	35	10,667	3,771	8,781	12,552
33	35	11,000	3,830	9,085	12,915
34	34	11,333	3,887	9,390	13,277
35	34	11,667	3,944	9,695	13,639
36	33	12,000	4,000	10,000	14,000
37	33	12,333	4,055	10,306	14,361
38	32	12,667	4,110	10,612	14,721
39	32	13,000	4,163	10,918	15,082
40	32	13,333	4,216	11,225	15,442
41	31	13,667	4,269	11,532	15,801
42	31	14,000	4,320	11,840	16,160
43	30	14,333	4,372	12,148	16,519
44	30	14,667	4,422	12,456	16,878
45	30	15,000	4,472	12,764	17,236
46	29	15,333	4,522	13,073	17,594
47	29	15,667	4,570	13,381	17,952
48	29	16,000	4,619	13,691	18,309
49	29	16,333	4,667	14,000	18,667
50	28	16,667	4,714	14,310	19,024

number cells counted	confidence interval %	cels/L (NUNC, 3mL)	total count error	min	max
51	28	17,000	4,761	14,620	19,380
52	28	17,333	4,807	14,930	19,737
53	27	17,667	4,853	15,240	20,093
54	27	18,000	4,899	15,551	20,449
55	27	18,333	4,944	15,861	20,805
56	27	18,667	4,989	16,172	21,161
57	26	19,000	5,033	16,483	21,517
58	26	19,333	5,077	16,795	21,872
59	26	19,667	5,121	17,106	22,227
60	26	20,000	5,164	17,418	22,582
61	26	20,333	5,207	17,730	22,937
62	25	20,667	5,249	18,042	23,291
63	25	21,000	5,292	18,354	23,646
64	25	21,333	5,333	18,667	24,000
65	25	21,667	5,375	18,979	24,354
66	25	22,000	5,416	19,292	24,708
67	24	22,333	5,457	19,605	25,062
68	24	22,667	5,497	19,918	25,415
69	24	23,000	5,538	20,231	25,769
70	24	23,333	5,578	20,544	26,122
71	24	23,667	5,617	20,858	26,475
72	24	24,000	5,657	21,172	26,828
73	23	24,333	5,696	21,485	27,181
74	23	24,667	5,735	21,799	27,534
75	23	25,000	5,774	22,113	27,887
76	23	25,333	5,812	22,427	28,239
77	23	25,667	5,850	22,742	28,592
78	23	26,000	5,888	23,056	28,944
79	23	26,333	5,925	23,371	29,296
80	22	26,667	5,963	23,685	29,648
81	22	27,000	6,000	24,000	30,000
82	22	27,333	6,037	24,315	30,352
83	22	27,667	6,074	24,630	30,703
84	22	28,000	6,110	24,945	31,055
85	22	28,333	6,146	25,260	31,407
86	22	28,667	6,182	25,575	31,758
87	21	29,000	6,218	25,891	32,109
88	21	29,333	6,254	26,206	32,460
89	21	29,667	6,289	26,522	32,811
90	21	30,000	6,325	26,838	33,162
91	21	30,333	6,360	27,154	33,513
92	21	30,667	6,394	27,469	33,864
93	21	31,000	6,429	27,785	34,215
94	21	31,333	6,464	28,102	34,565
95	21	31,667	6,498	28,418	34,916
96	20	32,000	6,532	28,734	35,266
97	20	32,333	6,566	29,050	35,616
98	20	32,667	6,600	29,367	35,966
99	20	33,000	6,633	29,683	36,317
100	20	33,333	6,667	30,000	36,667